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Development of humoral and cell-mediated immune responses was studied in guinea pigs infected with Coxiella burnetii administered in small-particle aerosols. Direct macrophage migration inhibition was observed in cultured peritoneal exudate cells as early as 3 days after exposure. Maximum inhibition of macrophages cultured with phase I or II antigen occurred 14 to 21 days post-exposure and persisted through 35 days. This inhibitory action was no longer detectable at 42 days. Serum antibody to the phase II antigen of C. burnetii →

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was detected at 14 days and to phase I antigen at 21 days, 18 days after the cell-mediated immune response.

Appearance of Cellular and Humoral Immunity to Guinea Pigs Following Infection with Coxiella burnetii Administered in Small-Particle Aerosols

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From the U. S. Army Medical Research Institute of Infectious Diseases

Frederick, Maryland

Running title: Early Immune Responses to C. burnetii Infection

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association of Accreditation of Laboratory Animal Care.

1 October 1976

ABSTRACT

Development of humoral and cell-mediated immune responses was studied in guinea pigs infected with Coxiella burnetii administered in small-particle aerosols. Direct macrophage migration inhibition was observed in cultured peritoneal exudate cells as early as 3 days after exposure. Maximum inhibition of macrophages cultured with phase I or II antigen occurred 14 to 21 days postexposure and persisted through 35 days. This inhibitory action was no longer detectable at 42 days. Serum antibody to the phase II antigen of C. burnetii was detected at 14 days and to phase I antigen at 21 days, 18 days after the cell-mediated immune response.

Previous in vitro studies indicated that homologous immune serum to phase I and II Coxiella burnetii not only enhanced the phagocytic uptake of rickettsiae (13,14, Kishimoto, R. A., B. J. Veltri, P. G. Canonico, F. G. Shirey and J. S. Walker, submitted for publication, 1976), but also potentiated their destruction within phagocytes (14, Kishimoto et al., submitted for publication). Although immune serum has not been shown to have a direct rickettsicidal action on C. burnetii, its passive transfer to nonimmune animals modifies the infection (1).

The mechanism(s) involved in acquired immunity to C. burnetii have not been studied extensively. Recent studies indicate that cell-mediated immunity (CMI) may play a role in C. burnetii infection. Peritoneal macrophages from guinea pigs previously immunized with phase I antigen are capable of killing phase I rickettsiae in the absence of homologous immune serum (14), and peripheral lymphocytes from humans exposed to C. burnetii up to 8 years earlier demonstrate marked lymphocyte transformation (LT) in vitro to both phase I and II C. burnetii antigens even in the absence of circulating antibody (12). Therefore, it appears that CMI may be required for protection against Q fever infection.

In the present study guinea pigs were infected with C. burnetii in small-particle aerosols to stimulate a natural respiratory exposure. Measurements of cellular and humoral parameters of immune response and its temporal development in infected animals were examined.

MATERIALS AND METHODS

Preparation of rickettsial stock suspension. The third egg passage (EP-3) of the Henzerling strain of C. burnetii in phase I was grown in chick embryo cells as previously described (14). The infectivity of the rickettsial suspension was estimated to be $10^{9.5}$ mouse median infectious doses ($MIPID_{50}$) after administration by the intraperitoneal (i.p.) route.

Guinea pigs. Outbred male Hartley strain guinea pigs, weighing approximately 350 to 450 g, were obtained from Buckberg Lab Animals, Tompkins Cove, N.Y. All animals were provided water and commercial guinea pig chow ad libitum.

Aerosol dissemination and animal exposure procedures. A total of 100 animals were exposed to 10^4 $MIPID_{50}$ of C. burnetii presented as small-particle aerosol (SPA). Aerosols composed of rickettsiae were generated with a Collison atomizer (16) into a Henderson apparatus (19) that was modified to include an exposure box suitable for simultaneous whole body exposure of 8 guinea pigs. The rickettsial suspension was diluted in Earle's 199 medium (E199) (GIBCO, Grand Island, N.Y.) prior to dissemination, and the concentration of rickettsiae was estimated during dissemination with samples collected in all-glass impingers (11) filled with E199. Animals were exposed to the aerosols for 10-min periods. The dose of rickettsiae inhaled (10^4 $MIPID_{50}$) by guinea pigs was computed on the basis of the estimated $MIPID_{50}$/L of aerosol X min respiratory volume of the guinea pig (0.11 L/min) (7) and the duration (min) of exposure. Thirty-two guinea pigs exposed to aerosols of sterile E199 medium served as controls. The rectal temperature and weights of 10 guinea pigs from each infected and control group were determined daily.

Inhibition of macrophage migration. Peritoneal exudate cells (PEC) were collected 4 days after i.p. injection of guinea pigs with 25 ml of sterile mineral oil (Marcol No. 90, Humble Oil and Refining Co., Houston, Texas). The PEC were harvested and processed according to the method of Harrington and Stastny (8). An agarose droplet method (8) was employed to detect the presence of direct macrophage migration inhibition. All cultures were maintained in E199 supplemented with 15% fetal calf serum (FCS). Twenty-seven replicate agarose droplets containing exudate cells were prepared for each sample. Sub-sets of 9 droplets each were overlayed with 0.2 ml of E199 supplemented with 15% FCS alone, or with 0.2 ml of medium containing either 2×10^7 formalin-killed particulate phase I (EP-3, Henzerling strain) or 4×10^6 phase II rickettsiae (EP-88, Nine-Mile strain). Cultures were incubated at 37 C for 24 h in 5% CO_2 and 95% humidified air. Exudate cells from noninfected guinea pigs were included as a control for antigen toxicity.

To estimate migration inhibition, agarose-cell droplets were examined with an inverted microscope fitted with a 0.5-mm grid in the ocular; the number of squares traversed by the margin of migrating cells was recorded. Inhibition of macrophage migration was calculated as follows:

$$\% \text{ migration inhibition} = 100 - \frac{\text{Mean area of migration with antigen}}{\text{Mean area of migration without antigen}} \times 100$$

Serological assays. Blood was collected from animals at weekly intervals and serum titrations were performed with the indirect immunofluorescent antibody technique of Bozeman and Elisberg (4) to evaluate antibody activity against phase I and II C. burnetii.

Necropsies and histopathological examinations. Four experimental and 2 control guinea pigs were necropsied at selected intervals for 29 days after infection. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

RESULTS

Clinical signs. Mean daily weight and temperature readings for 10 infected and 10 control animals are shown in Fig. 1. Onset of fever occurred 6 days after exposure with maximum readings on days 8 to 11; other overt clinical signs, such as roughened fur, lethargy, excessive thirst, and coryza, were noted 5 to 7 days postinfection, and signs persisted for 3 to 5 days. Infected animals failed to gain weight before termination of fever; subsequently, weight gain for the convalescent group paralleled that of the control group. Temperatures returned to base-line values by day 13. Control animals showed gradual increases in body weight and maintained constant body temperatures of 38 to 39 C throughout the period of observation.

Pathology. Interstitial pneumonia, detected after 5 days, became most pronounced and characterized by abundant exudation of fibrin, neutrophils, macrophages and lymphocytes in 9 to 11 days. Maximum development of lung lesions coincided with maximum temperature. Thirteen days after exposure, pneumonia with areas of consolidation still constituted the predominant lesion, with the infiltrate containing macrophages and lymphocytes. At 20 days pneumonitis became less pronounced; at 29 days lung lesions were minimal although macrophages and lymphocytes were still present. Granulomas of the liver and spleen were apparent at 9 days. Granulomatous splenitis was absent by 20 days, but hepatitis persisted for 29 days. Minimal lymphoreticular myocarditis and epicarditis were first observed on day 9.

Inhibition of macrophage migration. As early as 3 days after exposure, migration of macrophages was inhibited when peritoneal exudate cells from infected guinea pigs were cultured in phase I, but

not phase II antigen (Fig. 2). Macrophage inhibition was most pronounced at 14 and 21 days in the presence of either phase I or II antigen, and persisted at demonstrable levels until day 35. Reactivity, however, was no longer detectable 42 days postexposure. Simultaneous tests with macrophages from control guinea pigs and phase I or II C. burnetii antigen were found to be ± 5% of that observed for antigen-free cultures. Attempts to detect local induction of CMI in the respiratory tract at each sample time were unsuccessful, possibly due to the paucity of lymphoid cells.

Antibody response in infected guinea pigs. Indirect fluorescent antibody titers for sera from guinea pigs infected with C. burnetii are shown in Fig. 2. Antibody against phase II antigen, initially detected on day 14, was still present when the experiment was terminated at 42 days. Phase I antibody developing later than phase II antibody appeared at 21 days; maximum titers were achieved by day 28 and persisted throughout the 42-day observation period. Control guinea pigs developed no antibodies to either antigen.

DISCUSSION

These studies demonstrate that specifically sensitized populations of cells are present in peritoneal exudates of guinea pigs soon after their exposure to a small particle aerosol of C. burnetii. Macrophage migration inhibition was detected as early as 3 days postexposure, prior to overt clinical signs and well before any measurable humoral response. Macrophage migration inhibition was most marked at 14 days postexposure, shortly after maximum damage to lungs and other tissues was observed. This antigen-specific responsiveness of exudate cells

was still present on day 35 but was no longer detected at 42 days. These data are in agreement with Heggers et al. (9) who reported antigen-induced inhibition of migration of peritoneal macrophages obtained from guinea pigs on days 14 through 40 after infection with C. burnetii by the i.p. route, but they performed no assays prior to 14 days.

Histologically, the most pronounced lesions were severe interstitial pneumonia 9 to 11 days postexposure, granulomatous hepatitis and splenitis and minimal lymphoreticular myocarditis and epicarditis. Lesions in infected guinea pigs were similar to those reported in man (18). Our aerosol-infected animals developed more severe lung lesions and less severe liver lesions than those reported to occur in guinea pigs infected with C. burnetii by the i.p. route (10). Physical signs of infection, fever and weight loss, disappeared before phase I antibodies were detected. Although only phase II antibodies were detected at this time, host defense mechanisms were actively reversing the disease process.

The appearance of migration inhibition as early as 3 days after exposure is not unusual. Kostiala and McGregor (15) reported that in rats infected with Listeria monocytogenes, localization of MIF-producing cells in the peritoneal cavity coincided with the arrival on day 5 of circulating lymphocytes capable of protecting normal recipients against a Listeria challenge. Zinkernagel et al. (23) found that CMI activity appeared in the peritoneal cavity of mice 3 days after initiating a primary infection with Listeria.

Interactions between sensitized lymphocytes, released lymphokines such as MIF and macrophages and their role in host defense have not been elucidated. Bloom (3) suggests that MIF may participate in macrophage activation. Other investigators (6,17,20,21) have shown

that phagocytic, bacteriostatic, and even bactericidal activities of macrophages are enhanced in the presence of sensitized lymphocytes or their soluble products. In our previous study with guinea pigs immunized with killed phase I C. burnetii (Kishimoto et al., submitted for publication), migration inhibition developed at the same time the peritoneal macrophages became capable of killing phase I rickettsiae in vitro in the absence of immune serum. In studies by Jerrells and Hinrichs (Fed. Proc. 35:739, 1976, Abstract), intracellular growth of C. burnetii in cultures of normal guinea pig peritoneal macrophages was inhibited by immune lymphocytes, supernatant fluid from immune lymphocytes cultured with phase I antigen, the MIF-rich fraction of such cultures or with products of normal lymphocytes after stimulation with concanavalin A.

Unlike the CMI response, antibody production is slow to develop after vaccination or infection with C. burnetii; antibody directed against phase II antigens can be detected within 14 to 21 days and against phase I antigen by 34 to 35 days (5,22). In the present study, antibody against C. burnetii antigens was absent 3 to 7 days postexposure when activity of MIF was demonstrated, indicating that CMI is active and functioning well before humoral antibody is present. Other evidence also suggests that early protection against Q fever does not correlate with humoral antibody response; Benenson (2) found that although 50% of volunteers vaccinated with phase I Coxiella developed no detectable antibody, all were resistant to aerosol challenge with virulent phase I rickettsiae. However, persistence of antibody after detectable CMI activity suggests that both humoral and cellular elements are involved in resistance to C. burnetii infection.

We were unsuccessful in detecting local induction of CMI in the respiratory tract during infection. This may have been due to the lack of lymphoid cells in bronchoalveolar wash fluids. In continuing studies we are studying the role of other lymphokines in C. burnetii infections.

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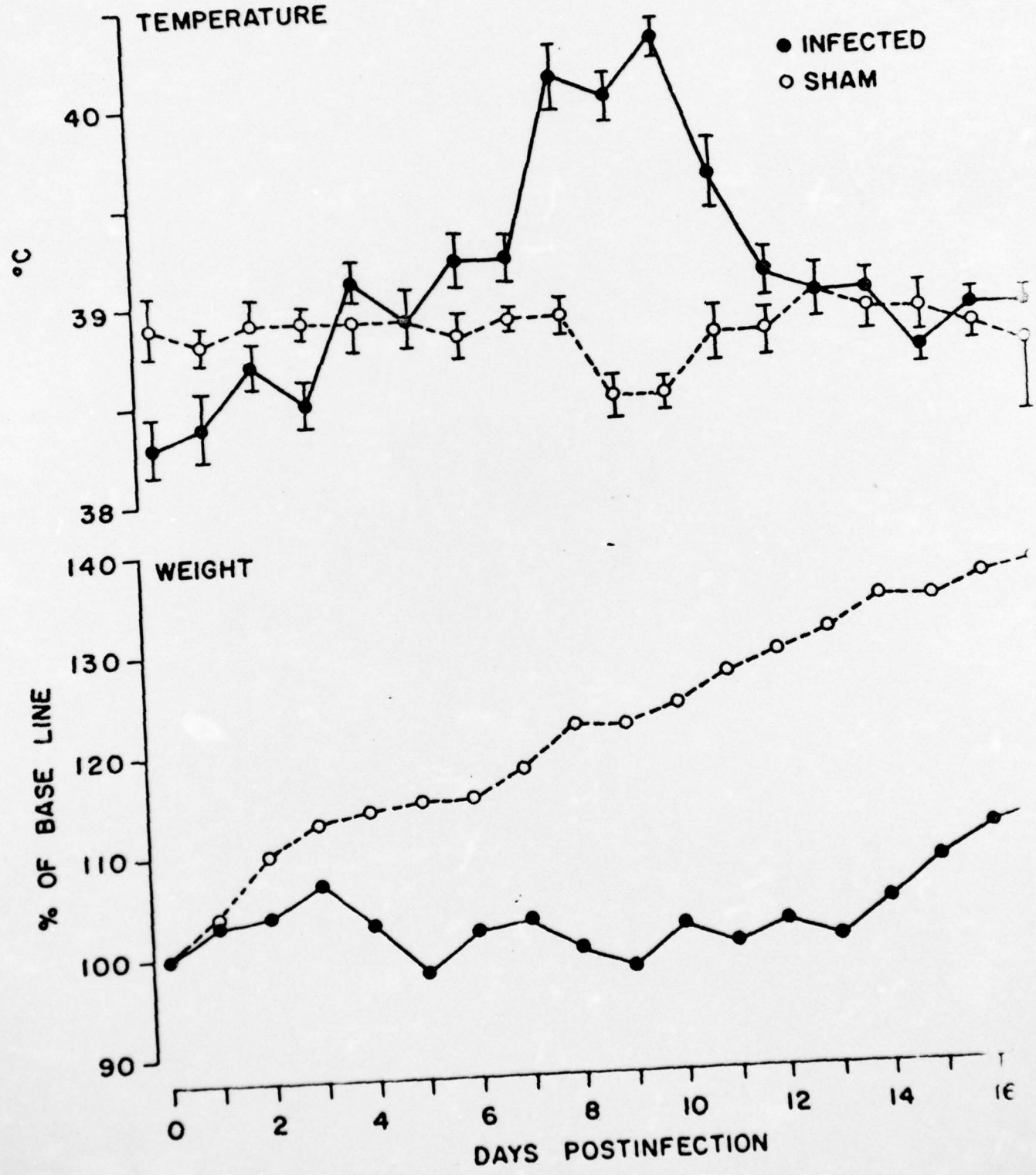
FIGURE LEGENDS

FIG. 1. Daily weight and temperature readings. Each point represents the mean ± standard error of the mean for 10 infected or sham-treated guinea pigs.

FIG. 2. Indirect fluorescent antibody titers and the percent macrophage migration inhibition of samples from guinea pigs infected with Coxiella burnetii. Each point represents the mean ± the standard error of the mean for 4 to 8 animals.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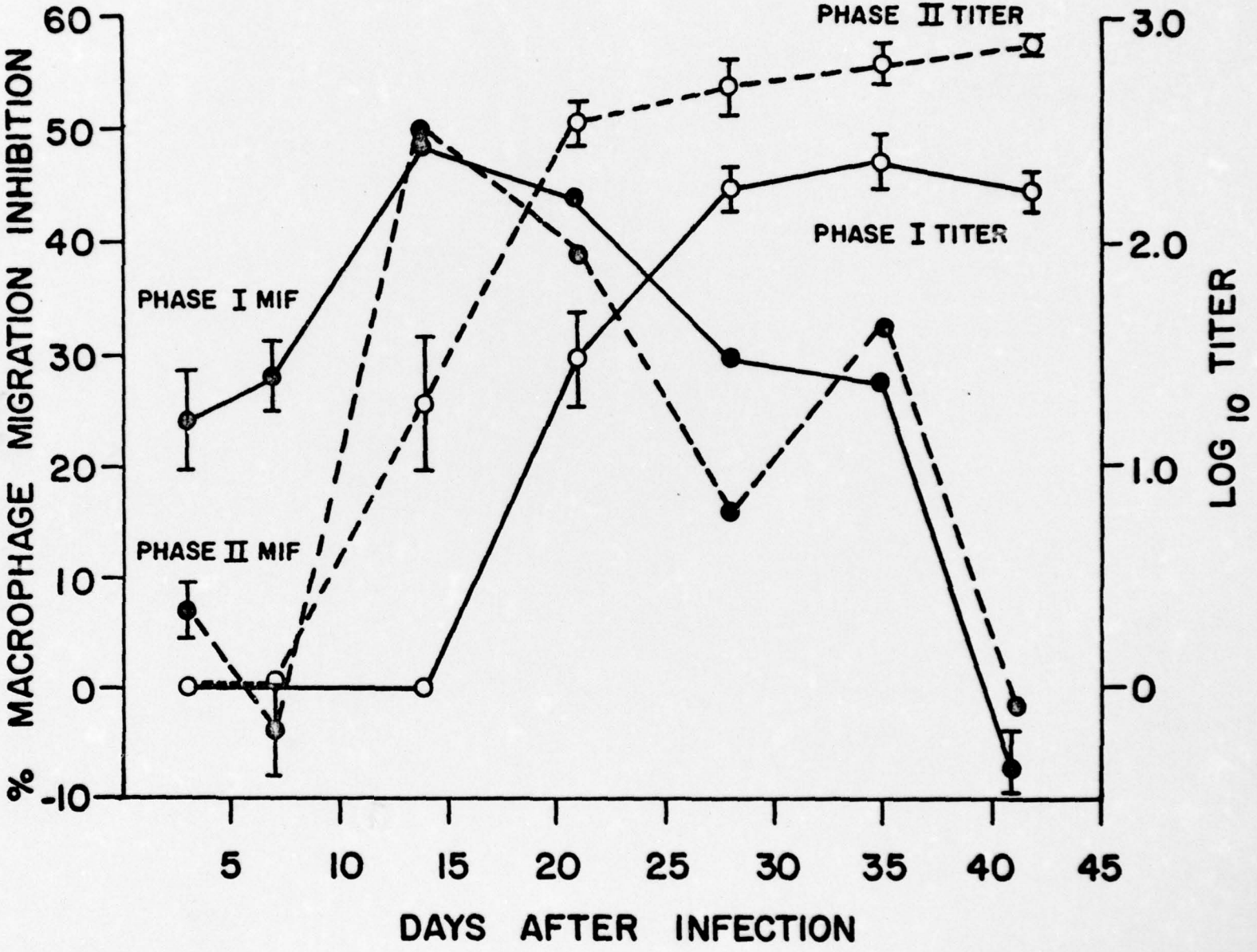
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